SELECTION OF A HIGH ANTHOCYANIN-PRODUCING CELL LINE FROM CALLUS CULTURES OF HYBRID POPLAR (*POPULUS ALBA* L. × *P. GLANDULOSA* UYEKI)

Myung Suk Choi[1] & Young Goo Park[2]

[1)] Division of Biotechnology, Forest Genetics Research Institute, P. O. Box 24, Kwonseon-gu, Suwon, 441–350, Korea
[2)] Department of Forestry, Kyungpook National University, Daegu, 702-701, Korea

Received December 20, 1996; accepted November 3, 1997

ABSTRACT

The reddish callus was visually selected from 15 callus lines based on color density. Anthocyanin synthesis ability was significantly different among 15 callus lines investigated. Line number 11 was considered as a reliable callus line for both high anthocyanin yield and rapid cell growth. Line number 11 frequently lost its ability for anthocyanin synthesis during repeated subculture and it did not provide reliable cell lines which produce high anthocyanin synthesis and cell growth. Callus derived from single cell colony was reselected by the cell aggregate selection method efficiently. The yield of anthocyanin from selected cell lines were increased almost two times (from 0.8 to 1.7) after the final selection (after 17 weeks). Anthocyanin content markedly increased from the 7th week but cell growth slightly decreased with selections. The content of anthocyanin from homogeneous callus lines varied from 0.8 to 1.7 per 0.5 g fresh weight of cultures.

Key words: *Populus alba* × *P. glandulosa*, anthocyanin, cell culture

INTRODUCTION

Plant secondary metabolites have been known to play a major role in the quality of food, favour and dye, and insecticide (VERPOORTE *et al.* 1993). Plant cell cultures could be an alternative approach to the production of such secondary metabolites. Although in the past years all economically important plants have been brought into cell culture, in most cases the productivity was too low to allow an economically feasible process (MISAWA 1988). Selection of stable cell line and finding of optimum conditions for cell growth can solve these problems (VERPOORTE *et al.* 1993). However, many compounds which would be of value have not yet been isolated in sufficiently high yield. The most widely used selection methods is not very great, and the productivity of a selected cell line often is unsuitable because cells with different productivity exist in the original aggregate (CONSTABEL *et al.* 1981).

PARK *et al.* (1992) reported that production medium and growth medium for two stage cultures was devised from this hybrid poplar. Selection by using two stage cultures are available for strains which is not easy the maintenance for long time.

Anthocyanins are major secondary metabolites that have been studied in this species so far. Banning of synthetic red dyes in food products stimulated interests in the development of pigments from natural sources. Although their primary role in the plant is related to coloration, roles in resistance to pathogens (WEISAETH, 1976) and pharmacological effects for man (BERETZ & CAZAHAVE 1988) also have been reported.

Hybrid poplar, *Populus alba* L. × *P. glandulosa* Uyeki, has been extensively planted in Korea since it was artificially bred by the Forest Genetics Research Institute of Korea in 1956. Those superior traits such as drought-resistance and fast growth than its parents made the hybrid a promising candidate for lumber production in short rotation in mountainous areas. Since cell culture can be established easily in this hybrid, it can serve as a model system for the production of useful secondary metabolites in tree species. The purpose of this study is to assess the problems of isolating stable, high yielding cell lines of *Populus alba* L. × *P. glandulosa* Uyeki.

MATERIALS AND METHODS

Visual selection

Callus derived from cambium tissues of *Populus alba* L. × *P. glandulosa* Uyeki were subcultured on MS (MURASHIGE & SKOOG 1962) medium containing 0.75% (w/v) agar, 3% (w/v) sucrose, 0.5 $mg \cdot l^{-1}$ 2,4-

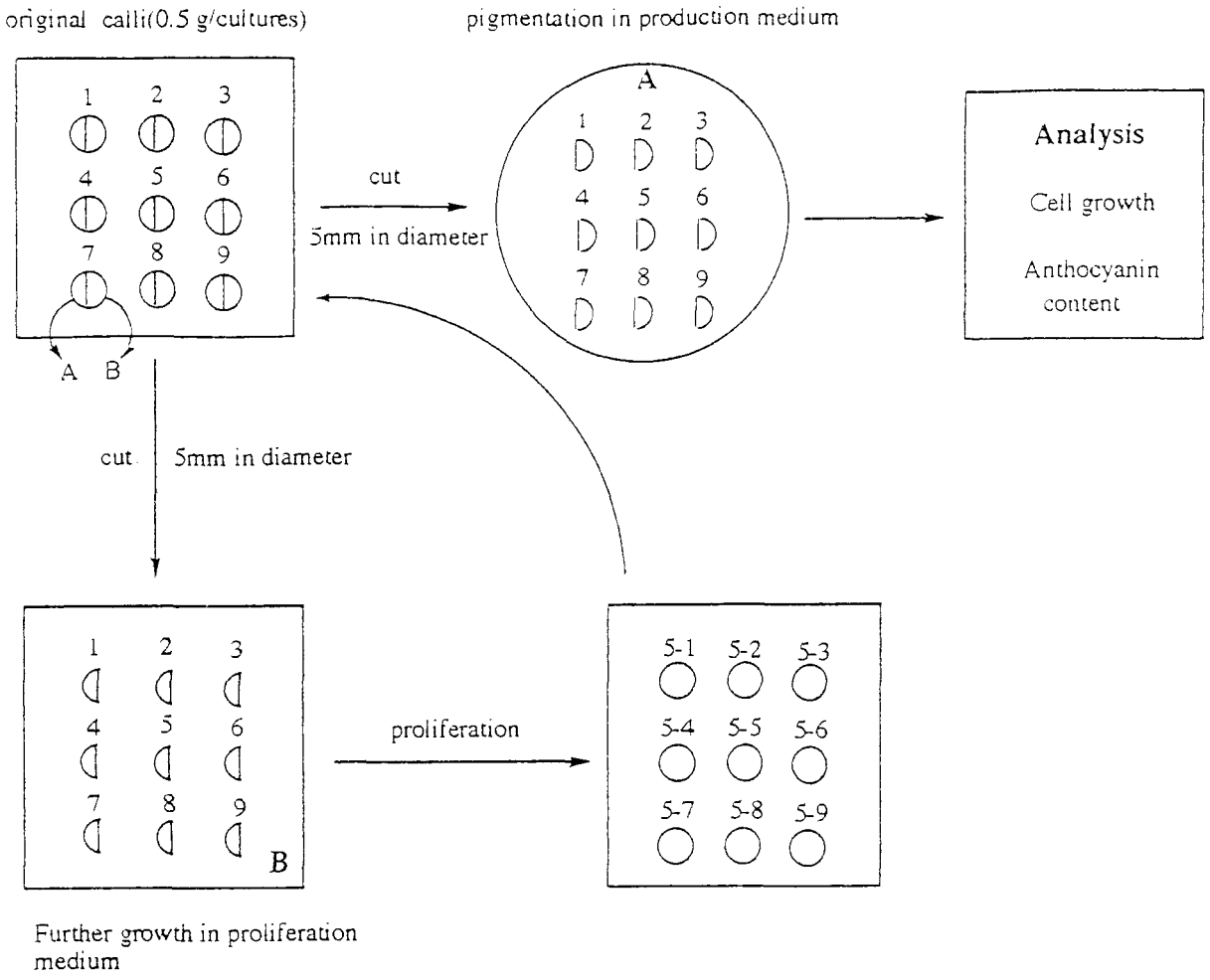

Figure 1. Schematic illustration of selection method of *Populus alba* × *P. glandulosa* Uyeki. Calli for anthocyanin synthesis (A) were cultured on MS agar medium supplemented with 1.0 mg·l^{-1} IAA, 1.0 mg·l^{-1} BA, and 5% sucrose under continuos illumination for 7 days. Calli for cell proliferation (B) were cultured on MS agar medium supplemented with 0.5 mg·l^{-1} 2,4–D, 0.1 mg·l^{-1} BA, and 3% sucrose under the dark condition.

dichlorophenoxyacetic acid (2,4-D), and 0.1 mg·l^{-1} benzylaminopurine(BA). For visual selection, each callus pieces were maintained at 25 °C with a photosynthetically active photon flux rate of 60 Em^{-2}·S^{-1} from cool white fluorescent tubes. Subcultures were made at 13 day intervals. Pigmented cells were counted under a microscope after separation of cells with 0.5% (w/v) cellulase "Onozuka R–10", 0.5% (w/v) Macerozyme (both from Kinki Yakult Ltd., Japan) and 0.02% (w/v) Pectolyase Y–23 (Seishin Ltd., Japan) as described previously (PARK & SON 1987). The anthocyanin content was determined by reading absorbance at 530 nm followed by the treatment reported by ARDITTE (1969).

Cell aggregate selection

Callus derived from single cell colony was reselected by the cell aggregate selection method as shown in Fig. 1. Callus was cut into two segments. Section A (ca. 5 mm in diameter) which was for anthocyanin synthesis was coded, arranged linearly, and then each segment was placed onto MS medium supplemented with 3% sucrose, 1.0 mg·l^{-1} indole-3-acetic acid (IAA), and 1.0 mg·l^{-1} BA (production medium) under fluorescent light of 60 Em^{-2}·S^{-1} . The other segment (section B), which was for cell proliferation was transferred onto a petri dish containing fresh MS medium supplemented with 0.5 mg·l^{-1} 2,4-D and 0.1 mg·l^{-1} BA (growth medium), and then cultured at 25 °C in the dark.

The section with the highest anthocyanin synthesis was selected by 17 successive clonal screening with 1 week interval as shown in Fig.1

Anthocyanin identification

Extraction and identification of anthocyanins were followed by the modified ARDITTI (1969) methods.

RESULTS AND DISCUSSION

The reddish callus was selected from 15 callus lines based on color density. Both optimal cell growth and anthocyanin synthesis were observed on the MS medium containing 0.5 mg·l^{-1} 2,4-D, 0.1 mg·l^{-1} BA, and 3% sucrose. Anthocyanin occurred in the central vacuoles, frequently disappearing in the callus cells. Anthocyanin yield and cell growth of each cell line are shown in Table 1. Anthocyanin synthesis ability was significantly different among 15 callus lines investigated. Line number 11 was considered as a reliable callus line for both high anthocyanin yield and rapid cell growth. In this cell line, more than 80% of the cells contained anthocyanin. In contrast, only 33% of the cells in callus line 4 were observed to contain anthocyanin(data not shown).

Visually selected callus line number 11 frequently lost its ability for anthocyanin synthesis during repeated subculture. It was thus not reliable cell lines for high anthocyanin synthesis and cell growth. After several subcultures, anthocyanin productivity of cells in the same clone became heterogeneous, maybe due to gene mutation and/or extrachromosomal variation observed in plastids of higher plants (MIZUKAMI *et al.*, 1987). This inconsistency can be improved by adopting cell aggregate selection.

The single cells were isolated by enzyme maceration from suspension cultured cells (Fig. 3.A). The first cell division of single cells was observed after 4 days of culture (Fig. 3.B), and calli were formed after 8 weeks

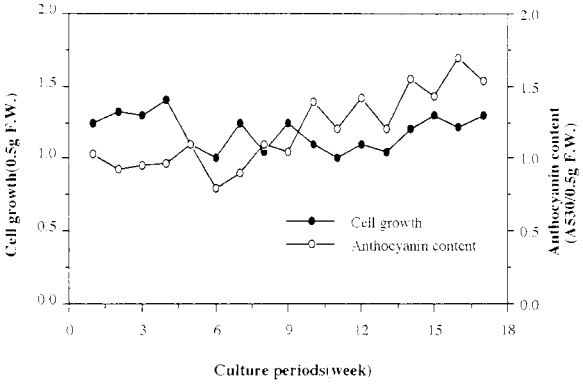

Figure 2. Changes in anthocyanin content during the serial subcultures *Populus alba* × *P. glandulosa* Uyeki. The callus for anthocyanin synthesis were cultured on MS agar medium supplemented with 0.1 mg·l^{-1} IAA, 1.0 mg·l^{-1} BA, and 5% sucrose under continuous illumination for 7 days. Each value represents the average of 5 replication, each in 3 independent experiments.

Table 1. Visual selection of a high anthocyanin producing calli lines of *Populus alba* L. × *P. glandulosa* Uyeki

Cell lines	Cell growth[1]	Color[3]	Anthocyanin content
1	++[2]	Y	1.614 ± 0.512
2	+	W	0.824 ± 0.745
3	+	Y.B	1.056 ± 0.353
4	+	W	0.756 ± 0.242
5	++	Y	1.359 ± 0.356
6	+	W	0.883 ± 0.374
7	++	Y	1.419 ± 0.462
8	+	B	0.987 ± 0.328
9	+	Y.B	1.558 ± 0.487
10	++	Y.B	1.201 ± 0.385
11	+++	Y	2.277 ± 0.367
12	++	Y.B	1.405 ± 0.481
13	+	Y	1.309 ± 0.373
14	++	Y.B	1.322 ± 0.292
15	+++	Y.B	1.095 ± 0.290

1) Calli were cultured on MS basal medium supplemented with 0.5 mg·l^{-1} 2,4-D, 0.1 mg·l^{-1} BA, and 3% sucrose under continuous illumination.
2) The number of + sign indicates the extent of cell growth (+: poor, ++: good, +++: very good).
3) The capital character represents the colors of the callus (Y: Yellow, W: White, Y.B: Yellowish Brown, B: Brown)

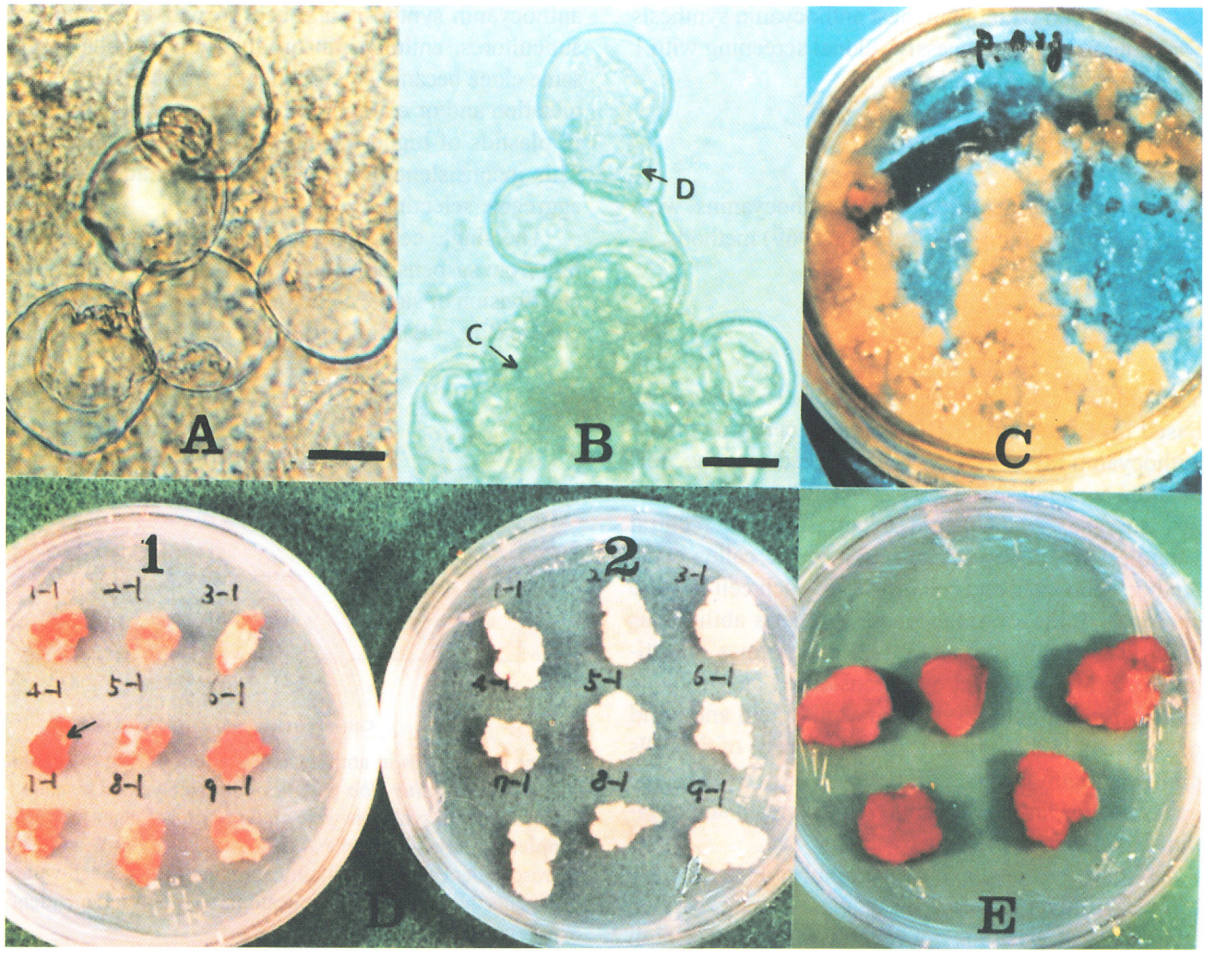

Figure 3. Schematic illustration of selection method of *Populus alba* × *P. glandulosa* Uyeki. **A** – Single cells of hybrid poplar; **B** – Cell division of single cell; **C** – Single cell-derived callus; **D-1** – Selection of high anthocyanin producing cell line using by small cell aggregate methods. The callus for anthocyanin synthesis was cultured on MS agar medium supplemented with 1.0 mg·l^{-1} IAA, 1.0 mg·l^{-1} BA, and 5% sucrose under continuous illumination for 7 days; **D-2** – Callus proliferation. The callus for cell proliferation cultured on MS agar medium supplemented with 0.5 mg·l^{-1} 2,4-D, 0.1 mg·l^{-1} BA, and 3% sucrose under dark condition; **E** – High anthocyanin-producing callus.

of culture (Fig. 3.C). Anthocyanin synthesis from single cell derived calli observed after 48–72 hrs of culture.

Pigmented cells were first detected between 24–48 hr after irradiation in section A. During subcultures of ection A, high growth rate and/or different pigmentation patterns (red, white, and mottled) were observed (Fig. 3.D-1). The high anthocyanin yielding callus segments selected from section A, were proliferated on the growth medium containing 0.5 mg·l^{-1} 2,4-D and 0.1 mg·l^{-1} BA in the dark as described above (Fig. 3.D-2). Once the frequency of pigmented cells reached maximum, callus aggregated and then cells eventually died. Although high yielding cell line was obtained, it was no reliable method for high yielding cell lines selection. Anthocyanin was accumulated in central vacuole of plant cells, was not the result of secretion to extracellular. Since viability of cells was decreased compared to non-pigmented cell lines. This selection method by two stage cultures can maintain the high yielding cell line for long time.

As shown in Fig. 2., the anthocyanin content of the callus tested fluctuated during subcultures. The yield of anthocyanin from given cells increased almost two times (from 0.8 to 1.7) after the final selection (after 17 weeks) (Fig. 3.E). Anthocyanin content increased markedly from the 7th week, but cell growth did slightly decreased with selections.

The content of anthocyanin from homogeneous callus lines varied from 0.8 to 1.7 per 0.5 g fresh weight of cultures. YAMADA *et al.* (1983) reported a similar result using homogeneous stable strains of high vitamin B_6 yielding cell lines. They obtained four times more secondary product than that of normal cell lines

(nonselected) of *Cytisus scoparius*. By the cell aggregate selection method, about two fold increment of yields was obtained. However, further experiments on the serial selection are still needed to warrant this system. Results described in this paper may help to overcome obstacles involved in plant secondary metabolite production such as low and instable productivity. And this study can serve as a model system in the study of useful secondary metabolites from tree species which is recalcitrant to in vitro manipulation. This system for selecting high anthocyanin yield cell lines appears to be readily adaptable for other tree species aimed at the commercially attractive biosynthesis of secondary metabolites.

ACKNOWLEDGEMENTS

This research was supported by a special grant from Korea Science & Engineering Foundation (KOSEP).

REFERENCES

ARDITTE, J. 1969: Floral anthocyanins in species and hybrids of *Broughtonia, Brassavola* and *Catteyopsis* (Corchicaceae). *Amer. J. Bot.* **59**:59–68.

BERETZ, A. & CAZAHAVE, J. P. 1988: The effect of flavonoids on blood vessel wall interactions. *In:* Plant Flavonoids in Biology and Medicine II, Cody, V., Middleton, E., Harborne, J. B., and Beretz, A. (Eds) Alan R. Liss, New York, pp. 187.

COSTABEL, F., RAMBOLD, S., CHATSON, K. B., KURZ, W. G. M. & KUTNEY, J. P. 1981: Alkaloid production in *Catharanthus roseous* (L.) G. Don. VI. Variation in alkaloid spectra of cell lines derived from one single leaf. *Plant Cell Rep.* **1**:3–5.

MISAWA, M. & NAKANISHIO, T. M. 1988: Antitumor compounds. Production by plant cell culture. *In*: Biotechnology in Agriculture and Forestry. Vol. 4. Medicinal and Aromatic Plants. Bajaj, Y. P. S. (ed). Springer-Verlag, Berlin, Heidelberg. pp 199–207.

MIZUKAMI, H., KONOSHIMA, H., & TABADA, M. 1978: Variation in pigment production in *Lithospermum erythorhizon* callus cultures. *Phytochemistry* **17**: 95–97.

MURASHIGE, T. & SKOOG, F. 1962: Revised medium for repid growth and bioassays with tabacco tissue cultures. *Physiol. Plant.* **15**:473–497.

PARK, Y. G. & SON, S. H. 1987: Protoplast isolation from callus and suspension cultured cells of *Populus alba*. *Korean J. Genetics* **9**:133–140.

PARK, Y. G., CHOI, M. S. & SON, S. H. 1992: Anthocyanin synthesis in cell cultures of *Populus alba* × *P. glandulosa* Uyeki. *J. Korean For. Soc.* **81**(2),183–190.

VERPOORTE, R., VAN DER HEIJDEN, R., HOGE, J. H. C. & TEN HOOPEN, H. J. G. 1993: *In*: Advances in Developmental Biology and Biotechnology of Higher Plants. Soh *et al.* (eds) pp 439–453.

WEISAETH, G. 1986: Quality problems in breeding for disease resistance in cabbage and cauliflower. *Qual. Plant Foods Hum. Nutr.* **26**, 167.

YAMADA, T., KATO, S., ISHIDA, K.K. & MINODA, Y. 1983: Production of anthocyanins by *Vitis* cell in suspension culture. *Agri. Biol. Chem.* **47**: 2185–2191.